The EMBO Journal vol.11 no.8 pp.2841 – 2847, 1992

# Stable surface expression of invariant chain prevents peptide presentation by HLA-DR

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Communicated by B.Dobberstein

**Class II major histocompatibility complex (MHC) molecules are cell surface glycoproteins that bind and present immunogenic peptides to T cells. Intracellularly, class II molecules associate with a polypeptide referred to as the invariant (Ii) chain. Ii is proteolytically degraded and dissociates from the class II complex prior to cell surface expression of the mature class II $\alpha\beta$ heterodimer. Using human fibroblasts transfected with HLA-DR1 and Ii cDNAs, we now demonstrate that truncation of the cytoplasmic domain of Ii results in the failure of Ii to dissociate from the $\alpha\beta$Ii complex and leads to stable expression of class II $\alpha\beta$Ii complexes on the cell surface. Furthermore, biochemical analysis and peptide presentation assays demonstrated that transfectants with stable surface $\alpha\beta$Ii complexes expressed very few free $\alpha\beta$ heterodimers at the surface and were very inefficient in their ability to present immunogenic peptides to T cells. These results support the hypothesis that the cytoplasmic domain of Ii is responsible for endosomal targeting of $\alpha\beta$Ii and directly demonstrate that association with Ii interferes with the antigen presentation function of class II molecules.**

*Key words*: antigen presentation/endosome/HLA-DR/intracellular traffic/invariant chain

## Introduction

The invariant chain (Ii) is a type II transmembrane glycoprotein associated intracellularly with class II major histocompatibility complex (MHC) molecules. Class II MHC molecules are expressed on antigen presenting cells and display peptides at the cell surface for recognition by $CD4^+$ T cells. The binding of peptide to class II MHC molecules is an intracellular event that requires uptake and processing of the antigen into an endocytic compartment (reviewed in Long, 1992). Although the function of Ii remained elusive for many years, recent findings suggest that Ii may play an important role in the antigen presentation function of class II MHC molecules. *In vitro* peptide binding studies have demonstrated that association with Ii significantly inhibits the ability of the human class II molecule HLA-DR to bind immunogenic peptides (Roche and Cresswell, 1990; Teyton *et al.*, 1990). These *in vitro* studies, and the fact that Ii is synthesized in excess over class II $\alpha$ and $\beta$ chains, suggest that newly synthesized class II molecules in the endoplasmic reticulum (ER) may be unable to acquire peptides for presentation to T cells. This is in complete contrast to class I molecules, whose assembly with $\beta_2$-microglobulin in the ER depends on the binding of specific peptides (Townsend *et al.*, 1990).

In addition, transient expression systems were used to show that Ii contains an intracellular sorting signal to target either free Ii or class II-associated Ii ($\alpha\beta$Ii) complexes to endosomal compartments (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Lamb *et al.*, 1991). The endosomal targeting signal has been attributed to sequences present in the N-terminal end of the cytoplasmic tail of Ii, since deletion of the first 15 or more residues from this region causes the Ii molecule to be excluded from endosomes and to be transported (possibly by a default transport pathway) to the cell surface (Bakke and Dobberstein, 1990). Alternatively, the N-terminal end could provide an endosomal retention signal, with the targeting signal located elsewhere on Ii, as suggested by a study using chimeric Ii–galactosyl transferase molecules (Nilsson *et al.*, 1991). Regardless of the mechanism, however, the targeting of class II molecules to endosomal compartments is thought to be crucial for class II function, as this process leads to the proteolytic degradation and dissociation of Ii from the $\alpha\beta$Ii complex (Blum and Cresswell, 1988).

The fate of $\alpha\beta$ chains associated with an Ii chain truncated in the cytoplasmic tail has not been studied and it is not known under what form these Ii molecules arrive at the cell surface. To address this, stably transfected human fibroblasts were generated that express HLA-DR $\alpha\beta$ heterodimers either alone, with intact Ii, or with truncated forms of Ii. Such transfected cells made it possible to analyze these molecules under steady-state conditions, and to follow their fate after biosynthetic labeling. Furthermore, because truncated Ii chains were stably expressed with $\alpha\beta$ heterodimers at the cell surface, it was possible to study the effect of Ii on DR-restricted T cell recognition.

## Results

### *Expression of HLA-DR and Ii in transfected fibroblasts*

Antigen presenting cells synthesize an excess of Ii over class II $\alpha\beta$ chains. To reproduce this situation in transfected cells, different transcriptional promoters in cDNA expression vectors were evaluated. The human fibroblast cell line M1, which is devoid of endogenous class II or Ii expression, was chosen as recipient. Pilot experiments demonstrated that the simian virus 40 (SV40) early promoter resulted in much lower expression than either the Rous sarcoma virus (RSV) promoter (Long *et al.*, 1991) or the cytomegalovirus (CMV) immediate early promoter (unpublished observations). M1 cells stably transfected with HLA-DRA and HLA-DRB



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cDNAs under the SV40 early promoter were isolated and cloned. Clone 4N5, stably expressing surface HLA-DR molecules, was able to present synthetic peptides to DR1-restricted T cells (see below). 4N5 cells were re-transfected with either full-length Ii (4N5Ii) or Ii constructs in which the first 15 or the first 20 amino acids of the cytoplasmic tail had been truncated (4N5IiΔ15 and 4N5IiΔ20), using vectors with RSV or CMV transcriptional promoters. Cells expressing surface Ii were isolated by cell sorting and enrichment with magnetic beads, and were maintained as populations of Ii-positive cells. After a pulse-labeling of the control B-LCL 45.1 or the fibroblast transfectants 4N5Ii or 4N5IiΔ15 for 20 min with [$^{35}$S]methionine, immunoprecipitation of all HLA-DR from these cells confirmed that Ii was synthesized in excess over class II molecules in all three cell types (Figure 1). This short pulse-label with methionine also demonstrated that the class II $\alpha$, $\beta$ and Ii chains assembled in the endoplasmic reticulum in these fibroblast transfectants.

As anticipated, expression of Ii in 4N5 did not significantly alter the level of cell surface expression of class II molecules (Figure 2). On the other hand, surface expression of Ii on these transfectants varied dramatically. Low levels of surface Ii were detected on 4N5Ii cells (Figure 2d), similar to the levels that have been observed in several class II-positive human cells (Koch *et al.*, 1991). By contrast, 4N5IiΔ15 and 4N5IiΔ20 cells express almost two orders of magnitude more surface Ii than 4N5Ii cells (Figure 2f and h). These results demonstrate that the truncated forms of Ii are efficiently transported to the cell surface, as observed previously in a transient expression system (Bakke and Dobberstein, 1990).

### *Surface expression of αβIi complexes*

The high surface levels of truncated Ii suggested that the half-life of these molecules may be relatively long. Experiments were therefore performed to determine the stability of class II and Ii molecules in these transfectants and to determine if the Ii molecules expressed on the surface of 4N5IiΔ20 were free or complexed with class II molecules. 4N5Ii and 4N5IiΔ20 cells were pulse-labeled with [$^{35}$S]methionine for 20 min and chased for up to 10 h in complete medium. Figure 3 demonstrates that Ii dissociated from the $\alpha\beta$Ii complex in the 4N5Ii transfectant within 4 h of biosynthesis. These kinetics of Ii dissociation are similar to those observed in the B-LCL 45.1 (data not shown) and those described previously in other B-LCL (Machamer and Cresswell, 1982). By contrast, the class II complexes isolated from the 4N5IiΔ20 transfectant contained large amounts of Ii even after 10 h of chase. Essentially identical results were obtained from pulse–chase studies of the 4N5IiΔ15 cell line (data not shown). The remarkable stability of the $\alpha\beta$Ii complexes after a long chase period with no evidence of proteolysis of Ii suggests that the complex has not traversed the endosomal compartment during intracellular transport.

To confirm that the $\alpha\beta$Ii complexes with truncated Ii were present on the cell surface, 4N5IiΔ15 cells were radiolabeled using [$^{125}$I]sulfosuccinimidyl (hydroxyphenyl)propionate. Figure 4 demonstrates that immunoprecipitation with an anti-DR$\alpha$ mAb revealed the presence of $\alpha$, $\beta$ and Ii chains on the surface of the 4N5IiΔ15 cell line. The series of spots associated with Ii observed here is due to sialic acid addition to the complex oligosaccharides present on the Ii polypeptide (Machamer and Cresswell, 1982). The results of this direct cell surface radioiodination experiment unambiguously

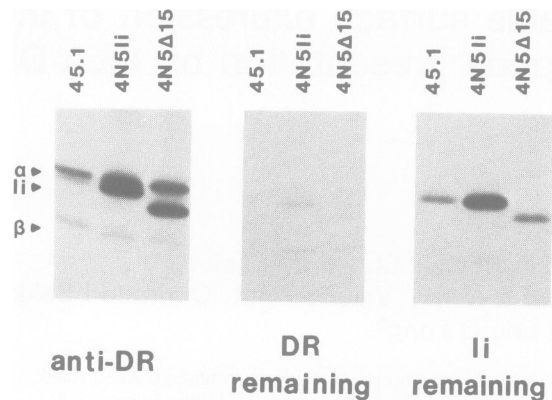


**Fig. 1.** Ii is synthesized in excess of HLA-DR in 45.1, 4N5Ii and 4N5IiΔ15. The B-LCL 45.1 or the fibroblast transfectants 4N5Ii or 4N5IiΔ15 were pulse-labeled with [$^{35}$S]methionine for 20 min and the class II molecules immunoprecipitated from cell lysates with the anti-DR$\alpha$ mAb DA6.147 (left panel). Following an additional treatment with this antibody, the remaining lysate was divided into equivalent aliquots which were then treated with the anti-DR$\alpha$ mAb DA6.147 (center panel) or the anti-Ii mAb POP.I (right panel) and analyzed by reducing SDS–PAGE and fluorography. The mobilities of the Ii chain as well as the DR$\alpha$ and DR$\beta$ chains are indicated. The intensity of the Ii chain is greater than the intensity of the DR$\alpha$ and DR$\beta$ chains because Ii contains many more methionine residues than either DR$\alpha$ or DR$\beta$. Note that Ii in the 4N5IiΔ15 cell migrates with a slightly greater electrophoretic mobility than does full-length Ii.

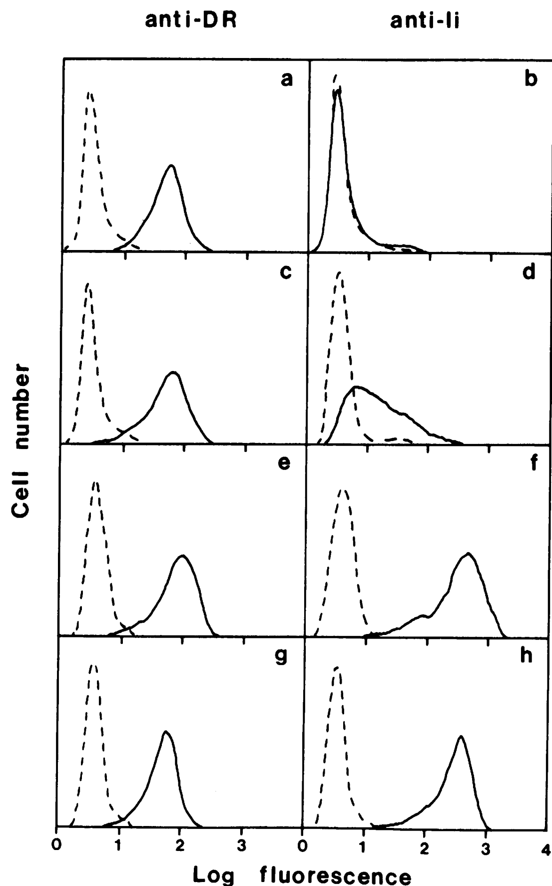


**Fig. 2.** Expression of HLA-DR and Ii on transfected fibroblasts. The cell surface expression of HLA-DR (left panels) and Ii (right panels) were assayed by FACScan analysis using the mAbs L243 and POP.I, respectively. The cell lines examined were 4N5 (**a** and **b**), 4N5Ii (**c** and **d**), 4N5IiΔ15 (**e** and **f**) and 4N5IiΔ20 (**g** and **h**). The dotted line in each panel represents the fluorescence profile of the FITC-conjugated reagent alone.

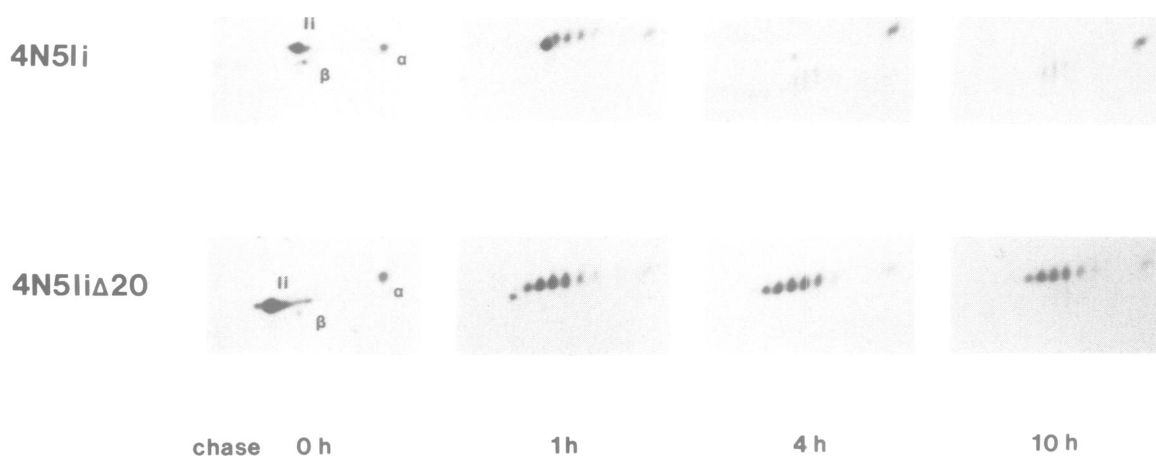


**Fig. 3.** Kinetics of Ii dissociation from $\alpha\beta$Ii in 4N5Ii and 4N5IiΔ20. The kinetics of Ii dissociation from the class II–Ii complexes present in 4N5Ii and 4N5IiΔ20 were examined by pulse–chase radiolabeling analysis. The cells were pulse-labeled for 20 min with [$^{35}$S]methionine, and chased in complete medium containing excess unlabeled methionine. At the indicated times of chase, aliquots of the reaction mixture were removed, lysed and the class II molecules immunoprecipitated with the anti-DR$\alpha$ mAb DA6.147. The samples were then analyzed by two-dimensional PAGE and the proteins visualized by fluorography.

demonstrate that deletion of the cytoplasmic tail of Ii causes class II molecules to escape a prolonged endosomal residence and to become transported efficiently to the cell surface as a stable $\alpha\beta$Ii complex.

### Surface expression of $\alpha\beta$Ii complexes and uncomplexed Ii

Since free Ii in cytoplasmic tail-deletion mutants has been shown previously to be transported efficiently to the cell surface, we performed experiments to determine whether or not free Ii existed on the surface of 4N5IiΔ20 in addition to Ii stably bound to class II $\alpha\beta$ heterodimers. 4N5IiΔ20 cells were pulse-labeled with [$^{35}$S]methionine for 30 min and the class II molecules were chased to the cell surface during a 4 h incubation in complete medium. After the chase period, all class II molecules were precipitated by two sequential incubations with an anti-DR$\alpha$ mAb. Following the second immunoprecipitation, the lysate was divided into equal aliquots and any remaining Ii was precipitated with an anti-DR$\alpha$ or an anti-Ii mAb. Figure 5 demonstrates that although sequential immunoprecipitations removed most cell surface class II molecules, a significant amount of free, completely glycosylated Ii remained on the surface of these cells. A parallel anti-DR$\beta$ precipitation confirmed that the anti-DR$\alpha$ reagent removed all of the surface class II molecules (data not shown). Note that the glycosylation pattern of the immunoprecipitated polypeptides is identical to that of the class II molecules isolated following direct cell surface radioiodination (Figure 4). To confirm that cell surface molecules were indeed precipitated, and to test for the stability of these $\alpha\beta$Ii complexes, the time of chase was increased to 18 h. Figure 5 also shows that essentially identical results were obtained following 18 h of chase, demonstrating that both free and class II-complexed Ii molecules were extremely stable when expressed on the surface of these cells.

It was possible that the free Ii observed in 4N5IiΔ20 was generated by the liberation of Ii from cell surface $\alpha\beta$Ii complexes. If this were the case, we would also expect to find free $\alpha\beta$ heterodimers on the surface of these cells. To test this hypothesis, 4N5IiΔ20 cells were pulsed with [$^{35}$S]methionine and chased as described above. In these experiments, however, all cell surface Ii molecules were sequentially precipitated with an anti-Ii mAb prior to treatment of the remaining lysate with an anti-Ii or an anti-DR$\alpha$ mAb. Unlike the anti-DR$\alpha$ mAb, the anti-Ii reagent did not completely remove all Ii molecules from the cell lysate even after three sequential incubations. However, the relative proportions of the DR $\alpha$, $\beta$ and Ii chains precipitated by the anti-Ii mAb in the final treatment were similar to those of the anti-DR$\alpha$ precipitate, suggesting that the anti-DR$\alpha$ mAb was not binding detectable amounts of class II $\alpha\beta$ heterodimers in addition to $\alpha\beta$Ii complexes. Despite the relative inefficiency of the anti-Ii precipitations, it appears unlikely that significant amounts of free $\alpha\beta$ heterodimers exist on the surface of 4N5IiΔ20 cells.

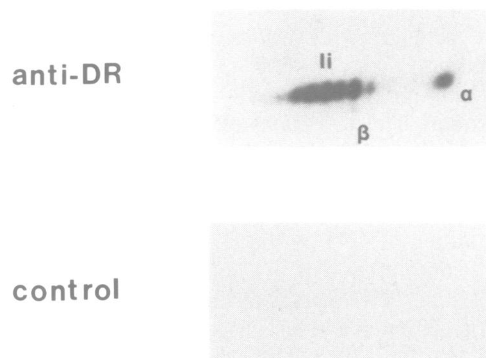


**Fig. 4.** Cell surface iodination of $\alpha\beta$Ii on 4N5IiΔ15. The presence of cell surface $\alpha\beta$Ii complexes on the 4N5IiΔ15 transfectant was examined by direct cell surface radioiodination and immunoprecipitation. Accessible amino groups on the surface of adherent 4N5IiΔ15 cells were radiolabeled with [$^{125}$I]sulfosuccinimidyl (hydroxyphenyl)propionate on ice for 1 h. The cells were then washed well, lysed and the radiolabeled (cell surface) class II molecules immunoprecipitated with the anti-DR$\alpha$ mAb DA6.147 (upper panel) or with a control ascites (lower panel).

### Peptide presentation by surface $\alpha\beta$Ii complexes

To test whether most of the class II molecules on the surface of 4N5IiΔ20 remained associated with Ii, the presence of functional cell surface $\alpha\beta$ heterodimers on 4N5IiΔ20 was assayed using a sensitive T cell readout. 4N5 and 4N5IiΔ20

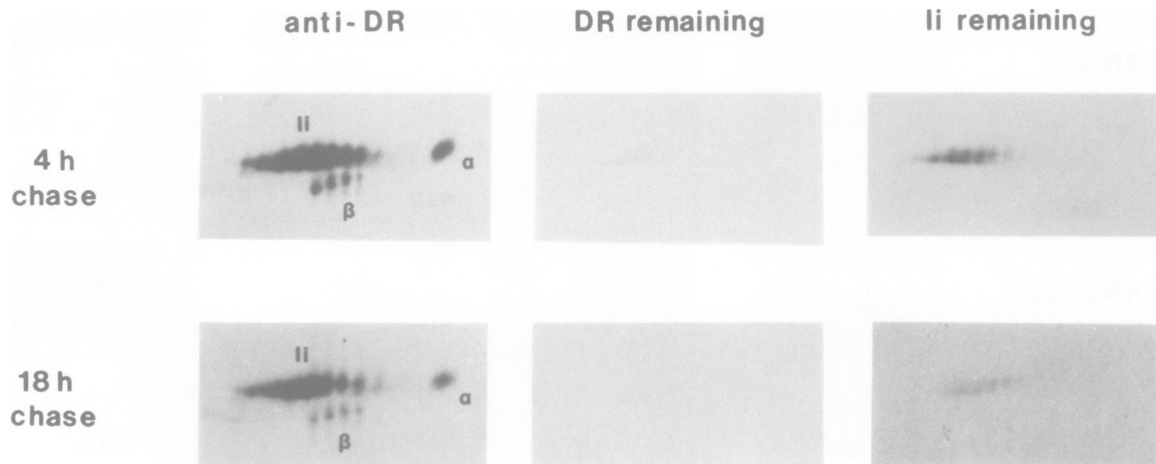


**Fig. 5.** Identification of cell surface αβIi and free Ii on 4N5IiΔ20. 4N5IiΔ20 cells were pulse-labeled with [$^{35}$S]methionine for 20 min and chased in complete medium containing excess unlabeled methionine for either 4 or 18 h. The cells were then lysed and the class II molecules were immunoprecipitated with the anti-DRα mAb DA6.147 (left panel). Following an additional treatment with this antibody, the remaining lysate was divided into equivalent aliquots which were then treated with the anti-DRα mAb DA6.147 (center panel) or the anti-Ii mAb POP.I (right panel). The immunoprecipitates were then analyzed by two-dimensional PAGE and fluorography.

cells were incubated with various concentrations of peptides corresponding to residues 307–318 of the influenza virus H3 hemagglutinin or residues 18–29 of the influenza virus matrix protein. These peptides efficiently sensitize antigen presenting cells to lysis by DR1-restricted, CD4-positive, peptide-specific CTL. Figure 6 (upper panel) demonstrates that ~0.02 μg/ml of H3 (307–318) was required for half-maximal killing of 4N5 by the H3-specific CTL E1.9. By contrast, a concentration of ~1 μg/ml was required for half-maximal killing of 4N5IiΔ20 by this same CTL. A similar pattern of recognition and lysis was observed in experiments using the M1 (18–29) peptide and the M1-specific CTL 130.1C6 (lower panel). Because of the lower sensitivity of the M1-specific T cells, as compared with the H3-specific T cells, it was not possible to reach maximum lysis of 4N5IiΔ20 even at the highest peptide concentration tested. In agreement with a previous report (Peterson and Miller, 1990), we also found that 4N5Ii cells were less efficient than 4N5 in presenting peptide to the T cells (Table I). This is presumably due to occupancy of cell surface class II αβ heterodimers on 4N5Ii by high affinity peptides encountered during transport through endosomal compartments. However, it is clear that surface Ii expression is far more inhibitory for peptide presentation than endosomal targeting of αβ chains.

Since the results of this investigation suggest that class II molecules in 4N5IiΔ20 are not transported to endosomal compartments *en route* to the cell surface, it is more appropriate to compare peptide presentation by 4N5IiΔ20 with peptide presentation by 4N5. The 50-fold lower peptide presentation by 4N5IiΔ20 as compared with 4N5 confirms that 4N5IiΔ20 possesses few cell surface class II αβ heterodimers and also demonstrates that association with Ii prevents the ability of class II molecules to present immunogenic peptides to antigen-specific T cells.

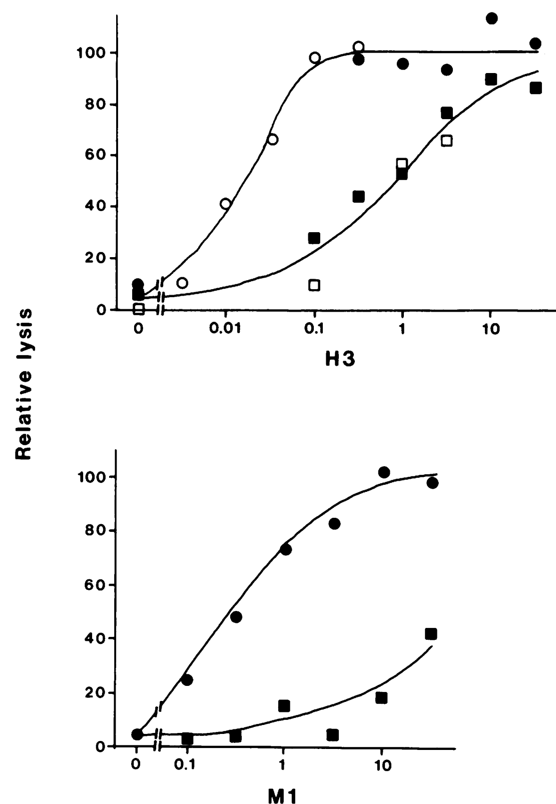


**Fig. 6.** Lysis of 4N5 and 4N5IiΔ20 by peptide-specific CD4$^+$ CTL. The ability of 4N5 and 4N5IiΔ20 to function as peptide presenting cells was examined by the use of peptide-specific, DR1-restricted CD4$^+$ CTL as described in Materials and methods. The relative lysis of either 4N5 (circles) or 4N5IiΔ20 (squares) by influenza virus H3 hemagglutinin peptide-specific CTL (upper panel) or influenza virus M1 matrix peptide-specific CTL (lower panel) was determined at various concentrations (μg/ml) of H3 or M1 peptide, respectively. The solid and open symbols represent the results obtained in two independent CTL assays. The results shown were obtained at an effector:target ratio of 12.5:1, and similar results were obtained at an effector:target ratio of 5:1 (not shown). Relative lysis is defined as the amount of lysis at any given peptide concentration represented as a percentage of the maximum specific lysis observed in the experiment (generally between 40 and 60%).

## Discussion

Previous studies have suggested that a signal present in the cytoplasmic tail of Ii was responsible for the targeting of

**Table I.** Relative peptide concentration required for lysis of different transfected cells

| Cell line | Peptide H3(307–318)[a] | M1(18–29)[b] |
|---|---|---|
| 4N5 | 1 | 1 |
| 4N5Ii | 6 | 3 |
| 4N5IiΔ20 | 50 | >30[c] |

These values were determined from experiments carried out simultaneously with the different cell lines. Since cytotoxic activity of T cells can vary between experiments the values listed represent the ratio of peptide concentration required for half-maximal lysis of 4N5Ii or 4N5IiΔ20 cells to that required for half-maximal lysis of 4N5 cells. A typical experiment is shown in Figure 6.
[a]Assayed with T cell line E1.9.
[b]Assayed with T cell line 130.1C6.
[c]A minimal estimate because maximal lysis could not be reached.

newly synthesized class II molecules to endosomal compartments (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Lamb *et al.*, 1991). This report extends these previous studies and further demonstrates that truncation of the cytoplasmic tail of Ii results in the stable surface expression of HLA-DR $\alpha\beta$Ii complexes. The surface expression of $\alpha\beta$Ii on the 4N5IiΔ15 and 4N5IiΔ20 cell lines (expressing HLA-DR $\alpha$ and $\beta$ chains in addition to a 15 or 20 amino acid deletion mutant of Ii, respectively) was examined by FACS analysis, pulse–chase metabolic labeling studies, and direct cell surface iodination. Immunoprecipitation experiments also confirmed that uncomplexed Ii was present on the surface of 4N5IiΔ20, a result which was not unexpected given that this cell synthesized Ii in excess of class II molecules and that such truncated Ii chains are not retained in the ER. Similar immunoprecipitation experiments also strongly suggested that these Ii molecules did not arise by the dissociation of $\alpha\beta$Ii complexes on the surface of these cells, as uncomplexed $\alpha\beta$ heterodimers could not be detected by this technique. The low level of free $\alpha\beta$ heterodimers on the surface of 4N5IiΔ20 cells was confirmed by the failure of the class II molecules on these cells efficiently to present immunogenic peptides to DR1-restricted, peptide-specific T cells. Taken together, these data demonstrate that the deletion of the cytoplasmic tail of Ii led to the transport of $\alpha\beta$Ii complexes to the cell surface and that these complexes remained stably associated.

Current evidence does not distinguish between the cytoplasmic tail of Ii acting as a direct endosomal targeting signal or as an endosomal retention signal for class II molecules. In the endosomal retention hypothesis, the actual targeting signal for routing $\alpha\beta$Ii complexes into endosomes would be distinct from a signal that causes the complex to be retained upon arrival in an endosomal compartment. While it is difficult to rule out such a hypothesis, the data presented here argue against such a mechanism. At the very least, they suggest that if $\alpha\beta$Ii complexes with truncated Ii chains were targeted to the endocytic pathway, it would have to be to early endosomes (which contain lower levels of proteolytic enzymes) from where these complexes would rapidly reach the cell surface. Previous work has demonstrated that Ii is extremely sensitive to the classes of proteinase likely to be encountered in endosomal compartments (Roche and Cresswell, 1991; Reyes *et al.*, 1991). As the Ii molecules present on the surface of 4N5IiΔ20 show no evidence of proteolytic degradation, it is highly unlikely that these molecules have passed through endosomal compartments for a significant period of time. It is thus more likely that the cytoplasmic tail of Ii does not serve as an endosomal retention signal, but instead plays a role in the direct targeting of class II molecules into endosomal compartments.

Although the experiments by Bakke and Dobberstein (1990) and Lotteau *et al.* (1990) suggested that the first 15 amino acids of the cytoplasmic tail of Ii are responsible for the endosomal localization of Ii in transient transfectants, at least two recent publications have challenged this hypothesis. In one study, a chimeric molecule composed of the Ii transmembrane and lumenal domains fused to the cytoplasmic tail of $\beta$-1,4-galactosyltransferase (normally retained in the Golgi apparatus) was targeted to post-Golgi 'endosomal' compartments which were indistinguishable from those that contained native Ii (Nilsson *et al.*, 1991). These data suggest that the transport signal in Ii is complex although another explanation, consistent with the data presented here, is that targeting of this chimeric protein may be due to the presence of an endocytosis signal in the cytoplasmic tail of the galactosyltransferase molecule.

In another study, Salamero *et al.* (1990) have suggested that class II molecules in L cell transfectants are transported directly from the Golgi apparatus to endosomal compartments even in the absence of Ii. This hypothesis was based on the observation that class II molecules could be iodinated *in situ* in endosomal compartments even in cells which failed to internalize their class II molecules. However, it is possible that the iodinated class II molecules arose from molecules which were very slowly internalized and had accumulated at steady state in the endosomal pathway. As mentioned above, the sensitivity of Ii to proteolysis in endosomal compartments and the observed stability of $\alpha\beta$Ii complexes in 4N5IiΔ20 cells are inconsistent with a targeting signal located outside of the 15 amino acid cytoplasmic tail of Ii. Rather, the data presented here strongly support the hypothesis that the cytoplasmic tail of Ii itself is the signal responsible for the targeting of class II molecules to endosomes.

As shown here, cells expressing class II molecules stably associated with Ii function poorly as peptide presenting cells. The presence of Ii may inhibit the ability of class II molecules to present peptides to T cells by different mechanisms. Previous *in vitro* studies demonstrated that Ii association signifcantly inhibits the ability of class II molecules to bind immunogenic peptides (Roche and Cresswell, 1990,1991; Teyton *et al.*, 1990). We now demonstrate for the first time that $\alpha\beta$Ii complexes expressed on the surface of viable antigen presenting cells are deficient in their ability to present immunogenic peptides to T cells. The poor presentation of peptide by 4N5IiΔ20 cells could easily be explained if the class II molecules on 4N5IiΔ20 were incapable of binding the immunogenic peptide. However, it is also possible that the $\alpha\beta$Ii complexes on the surface of 4N5IiΔ20 have bound the peptide, but that the inhibition of peptide presentation was due to steric interference by Ii with the CD4– or T cell receptor–class II interactions. The availability of transfectants expressing stable $\alpha\beta$Ii complexes at the cell surface will provide a powerful tool to investigate how the structure of these complexes may interfere with normal class II functions.

## Materials and methods

### Plasmids

The major form of Ii expressed in human cells has an apparent molecular weight of 33 000 (p33). Other minor forms of Ii exist, due to an alternative splicing (p41), and to an alternative translation initiation codon (p35; Strubin *et al.*, 1986a,b). All the experiments reported here were carried out with the major p33 form (hereafter referred to as Ii) or truncated derivatives of p33. cDNAs encoding different forms of Ii in the vector pSVL (Bakke and Dobberstein, 1990) were inserted into vectors suitable for stable expression in human cells. The full-length Ii p33 cDNA was excised with *Sma*I−*Bam*HI and blunt-end ligated into the *Xba*I-digested vector CDM8 (Aruffo and Seed, 1987), after filling the protruding ends with Klenow polymerase. cDNA expression in CDM8 is under the control of the human cytomegalovirus (CMV) immediate early promoter. A cDNA missing the first 15 codons of Ii (IiΔ15) was excised with *Sal*I(filled)−*Bam*HI and ligated into the *Sal*I(filled)−*Bam*HI digested vector RSV.5(gpt) (Long *et al.*, 1991). Amino acid 16 in Ii is a methionine and provides the translational start in IiΔ15. cDNA expression in RSV.5(gpt) is under the control of the long terminal repeat of Rous sarcoma virus (RSV). A fragment from pSV2-gpt is present in RSV.5(gpt) to provide selection for stably transfected cells. A cDNA missing codons 2−20 of Ii (IiΔ20) was cloned into RSV.5(gpt) as described above for IiΔ15. Full-length cDNA clones for the DRα and DRβ chains, under the control of the SV40 early promoter, have been described (Tonnelle *et al.*, 1985; Sekaly *et al.*, 1986).

### Human fibroblast transfectants

The human fibroblast cell line M1 (Royer-Pokora *et al.*, 1984) was co-transfected with HLA-DRA and HLA-DRB cDNAs, and pSV2-neo for selection, as described (Long *et al.*, 1991). After sorting of the brightest DR-positive cells by flow cytometry and cloning by single cell sorting, clone 4N5 was obtained. 4N5 cells were re-transfected with CDM8-Ii and pSV2-gpt (Mulligan and Berg, 1981), RSV.5(gpt)-IiΔ15 and RSV.5(gpt)-IiΔ20. Transfected cells were selected as described (Long *et al.*, 1991) and cells expressing invariant chain were enriched with magnetic beads coupled with goat anti-rabbit Ig (Dynal, Great Neck, NY), after binding the mouse IgM anti-Ii POP.I4.3 (Marks *et al.*, 1990) to the cells, followed by rabbit anti-mouse IgM. The fibroblast transfectants were maintained in a Dulbecco's Modified Eagle's Medium supplemented with 10 mM HEPES, pH 7.4, 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 10 μg/ml gentamicin sulfate and 0.25 mg/ml G418 (active ingredient, Geneticin, Gibco-BRL) for selection. For Ii transfectants only, the additional selection reagents mycophenolic acid (10 μg/ml) and xanthine (100 μg/ml) were added. The adherent transfectants were grown to no more than 75% confluence in 75 $cm^2$ tissue culture flasks and passaged by trypsinization. Cell surface expression of HLA-DR on the fibroblast transfectants was determined with a FACScan (Becton-Dickinson) after staining the cells with the anti-DR IgG mAb L243 (American Type Culture Collection) and a FITC-conjugated goat anti-mouse IgG antibody. Cell surface expression of Ii was determined similarly with the anti-Ii IgM mAb POP.I, followed by rabbit anti-mouse IgM and FITC-conjugated goat anti-rabbit antibodies.

### Radiolabeling and immunoprecipitation

The human B-LCL 45.1 (Kavathas *et al.*, 1980) was metabolically labeled with $[^{35}S]$methionine as described previously (Machamer and Cresswell, 1982) and lysed using Triton X-100 as described below. Human fibroblast cell lines were metabolically labeled in suspension with $[^{35}S]$methionine following trypsinization. Briefly, adherent cells were harvested by trypsinization and cultured in methionine-free Dulbecco's Modified Eagle's Medium containing 5% dialyzed fetal calf serum at 37°C at $5\times10^6$ cells/ml. After 1 h, this medium was replaced and supplemented with 0.5 mCi $[^{35}S]$methionine. Following a 20 min 'pulse' label at 37°C, the cells were pelleted by centrifugation and re-suspended in 'complete' medium containing a 5-fold excess of methionine at $1\times10^6$ cells/ml and divided into equivalent aliquots. These aliquots were then incubated at 37°C for various lengths of time ('chased') prior to harvesting the cells by centrifugation, washing the cell pellet with 10 mM Tris, 150 mM NaCl, pH 7.4, and lysing the cells in wash buffer containing 1% Triton X-100 and proteinase inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM iodoacetamide and 10 μg/ml $\alpha_2$-macroglobulin) for 1 h on ice at $20\times10^6$ cells/ml.

The cell surface proteins of adherent fibroblasts were radiolabeled with $[^{125}I]$sulfosuccinimidyl (hydroxyphenyl)propionate as described (Thompson *et al.*, 1987). Briefly, a 75% confluent tissue culture flask (75 $cm^2$) containing fibroblasts was labeled with 0.75 mCi $[^{125}I]$sulfosuccinimidyl (hydroxyphenyl)propionate (Pierce Chemical Co.) in 3 ml of Hanks' Balanced Salt Solution for 1 h on ice. The labeling reaction was then terminated by the addition of 10 mM Tris, pH 7.3 and the adherent cells were washed and lysed as described above. Class II molecules were immunoprecipitated from cell lysates using anti-class II mAbs and Protein A−Sepharose as described previously (Marks *et al.*, 1990; Machamer and Cresswell, 1982). Immunoprecipitates were analyzed by two-dimensional polyacrylamide gel electrophoresis (non-equilibrium pH gradient electrophoresis followed by reducing SDS−PAGE) as described (Machamer and Cresswell, 1982). The radioactivity present in the samples was visualized by fluorography. The anti-DRα mAb DA6.147 (Guy *et al.*, 1982) and the anti-Ii mAb POP.I were used as unpurifed ascites and were the generous gift of Dr Peter Cresswell, Yale University School of Medicine, New Haven, CT.

### Cytotoxicity assay

Peptide presenting ability of the class II transfectants was assayed using peptide-specific, HLA-DR1 restricted CD4-positive CTL using a standard $^{51}Cr$ release assay. Briefly, adherent fibroblasts were labeled overnight with $Na^{51}CrO_4$ and unincorporated label was removed by washing the cells in media. The cells were then removed by trypsinization, washed and incubated with various concentrations of a peptide corresponding to residues 307−318 of the influenza virus hemagglutinin [H3 (307−318)] or to a peptide corresponding to residues 18−29 of the influenza virus matrix protein [M1 (18−29)]. Following incubation with the peptide for 3 h, $2.5\times10^3$ live target cells were added to the DR1-restricted, CD4-positive, H3-specific CTL E1.9 (Karp and Long, 1992) or the M1-specific CTL 130.1C6 (Jaraquemada *et al.*, 1990) at an effector:target ratio of 12.5:1 in V-bottomed 96-well plates. After incubation for 8 h, supernatants were harvested and counted for $^{51}Cr$ release as described previously (Karp and Long, 1992).

## Acknowledgements

The authors wish to thank Drs William Biddison for the CTL 130.1C6, Peter Cresswell for monoclonal antibodies, Robert DeMars for the B-LCL 45.1, Dolores Jaraquemada for generating the 4N5 cell line while in this laboratory, Salvatore Pizzo for human $\alpha_2$-macroglobulin and Brian Seed for plasmid CDM8.

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*Received on February 24, 1992; revised on April 13, 1992*